Differential Contributions of *Caenorhabditis elegans* Histone Deacetylases to Huntington Polyglutamine Toxicity

Emily A. Bates,1,2 Martin Victor,1 Adriana K. Jones,2 Yang Shi,1 and Anne C. Hart1,2

1Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115, and 2Massachusetts General Hospital, Center for Cancer Research, Charlestown, Massachusetts 02129

Expansion of a polyglutamine tract in the huntingtin protein causes neuronal degeneration and death in Huntington’s disease patients, but the molecular mechanisms underlying polyglutamine-mediated cell death remain unclear. Previous studies suggest that expanded polyglutamine tracts alter transcription by sequestering glutamine rich transcriptional regulatory proteins, thereby perturbing their function. We tested this hypothesis in *Caenorhabditis elegans* neurons expressing a human huntingtin fragment with an expanded polyglutamine tract (Htn-Q150). Loss of function alleles and RNA interference (RNAi) were used to examine contributions of *C. elegans* cAMP response element-binding protein (CREB), CREB binding protein (CBP), and histone deacetylases (HDACs) to polyglutamine-induced neurodegeneration. Deletion of CREB (*crh-1*) or loss of one copy of CBP (*cbp-1*) enhanced polyglutamine toxicity in *C. elegans* neurons. Loss of function alleles and RNAi were then used to systematically reduce function of each *C. elegans* HDAC. Generally, knockdown of individual *C. elegans* HDACs enhanced Htn-Q150 toxicity, but knockdown of *C. elegans* hda-3 suppressed toxicity. Neuronal expression of *hda-3* restored Htn-Q150 toxicity and suggested that *C. elegans* HDAC3 (HDA-3) acts within neurons to promote degeneration in response to Htn-Q150. Genetic epistasis experiments suggested that HDA-3 and CRH-1 (*C. elegans* CREB homolog) directly oppose each other in regulating transcription of genes involved in polyglutamine toxicity. *hda-3* loss of function failed to suppress increased neurodegeneration in hda-1/+;Htn-Q150 animals, indicating that HDA-1 and HDA-3 have different targets with opposing effects on polyglutamine toxicity. Our results suggest that polyglutamine expansions perturb transcription of CREB/CBP targets and that specific targeting of HDACs will be useful in reducing associated neurodegeneration.

Key words: Huntington; polyglutamine; HDAC; polyQ; neurodegeneration; CBP

Introduction

Huntington’s disease (HD) is one of several neurodegenerative diseases caused by expansion of a polyglutamine (polyQ) tract (Zoghbi and Orr, 2000; Ross, 2002). In Huntington’s disease, the expansion occurs in the huntingtin protein. Understanding the primary changes that occur in neurons expressing expanded huntingtin could lead to therapeutic interventions. Several hypotheses have been proposed to explain how expansions of polyglutamine domains lead to neurodegeneration. Although, multiple pathways likely contribute to neuronal death, many studies suggest that expanded polyglutamine domains interfere with transcriptional regulation. In patient tissue, as well as in cell culture, vertebrate, and invertebrate models, the expanded polyglutamine form of huntingtin protein binds the acetyltransferase histone (CBP); both huntingtin and CBP are sequestered into aggregates (McCampbell et al., 2000; Nucifora et al., 2001; Steffan et al., 2001; Jiang et al., 2003). Moreover, acetyltransferase activity is reduced in cell culture models of Huntington’s disease (Nucifora et al., 2001). Increased CBP expression restores histone acetylation, altered transcription, and retinal degeneration in a *Drosophila* model of polyglutamine disease (Taylor et al., 2003), supporting the hypothesis that CBP is a critical target of expanded polyQ tracts.

Histone acetyltransferase activity is directly opposed by histone deacetylases (HDACs), which remove acetyl groups from core histones. HDACs are divided into three classes based on structure and function. Class I and class II HDACs are similar in sequence and are part of numerous repressor complexes (Grozinger et al., 1999; Knoepfler and Eisenman, 1999). Class III HDACs share little homology with class I and class II HDACs and are homologous to the NAD+-dependent yeast HDAC, SIR2 (silent information regulator 2) (Brachmann et al., 1995), and are implicated in chromatin silencing, cellular metabolism, and aging (Guarente, 2000; Tanner et al., 2000). HDAC proteins have diverse functions and targets, suggesting that inhibition of individual proteins could have different effects on neuronal survival and resistance to polyglutamine toxicity.

Chemical inhibitors of class I and class II HDACs reduce degeneration in cell culture, yeast, *Caenorhabditis elegans*, and *Drosophila* models of polyglutamine toxicity (McCampbell et al.,
2001; Steffan et al., 2001; Hockly et al., 2003; Parker et al., 2005). One HDAC inhibitor, suberoylanilide hydroxamic acid, partially rescues motor defects in a mouse model of Huntington’s disease (Hockly et al., 2003). However, chemical inhibitors of enzymes are not necessarily specific. Most HDAC inhibitors indiscriminately target class I and class II HDACs, complicating the identification of critical targets.

Our C. elegans model expresses exon 1 of human Htn with an expanded polyglutamine (Htn-Q150) reminiscent of the R6/2 mouse model of HD (Mangiarini et al., 1996; Faber et al., 1999). Expression of Htn-Q150 in the ASH sensory neurons causes progressive neurodegeneration. As in patients, aggregation of mutant protein and neurodegeneration are directly related to the length of the polyglutamine tract and age of the animal (Faber et al., 1999). We found that loss of CBP and CREB function increases polyglutamine toxicity. We surveyed C. elegans HDACs to address possible differential contributions to Htn-Q150 toxicity in neurons and found that C. elegans HDACs have divergent effects on polyglutamine toxicity.

Materials and Methods

RNA interference. To target individual C. elegans HDACs and decrease off-target effects, unique fragments of HDAC exonic sequences were amplified by PCR from genomic DNA using primers listed in the supplemental material (supplemental Table 1, available at www.jneurosci.org). Restriction sites were designed into primers when necessary for cloning into the L4440 plasmid (Timmons et al., 2001). Constructs were amplified in DH5α cells, and HT115 bacteria were transformed to yield strains for feeding RNA interference (RNAi). RNAi feeding strains for hda-1 (C53A5.3), hda-2 (C08B11.2), and hda-3 (R06G1.1) were provided by Iva Greenwald (Columbia University, New York, NY) (Jarrar and Greenwald, 2002). RNAi feeding strain for hda-10 (Y51H1A.5) was provided by Mark Vidal (Harvard Medical School, Boston, MA). All bacteria feeding strains were cultured as described previously to induce expression of double-stranded RNA (dsRNA) (Timmons et al., 2001). Gravid adult hermaphrodites were lyzed using a 20% bleach and 2.5% 10N NaOH solution to release eggs of similar ages. Larvae (stage L1) from these egg preparations were placed onto RNAi bacterial strains and passedaged to new RNAi plates daily starting at young adult stage. On the eighth day, animals were washed off plates with M9 buffer, dye filled with diocadecyl tetramethylindocarbocyanine (DiD) (Perkins et al., 1986), and allowed to recover for at least 20 min before scoring.

To confirm ASH neuron sensitivity to RNAi, green fluorescent protein (GFP) fluorescence was visually assessed in rtls11/osm-10:Htn-Q150, osm-10::GFP) young adult animals that were fed bacteria expressing GFP dsRNA from L1 stage. In addition, degeneration of ASH neurons was assessed in 8- and 9-day-old rtls11/osm-10:Htn-Q150, osm-10::GFP) fed bacteria expressing pqa-1 (Faber et al., 2002) dsRNA starting at L1 stage.

Strains and aging. Animals were cultured at 25°C using standard methods unless otherwise indicated (Brenner, 1974). rtls18[Htn-Q150], rtls14[Htn-Q150], and rtls11[Htn-Q150] transgenic animals express osm-10::Htn-Q150 as described previously (Faber et al., 1999). sir-2.1(0Ex) are animals that carry the integrated array gen3[sir-2.1] (Tissenbaum and Guarente, 2001). deg-1[rt70] (Faber et al., 2002) animals were aged to 4 d at 20°C when 9 ± 4% of the ASH neurons degenerate. For each genetic strain generated, control lines [lacking loss of function (I) mutation or Htn-Q150 transgene] were scored in parallel. To look for suppression of nectroc neuronal death, deg-1[rt70] animals were aged to 7 d at 15°C when 78 ± 4% of the ASH neurons degenerate. After reaching reproductive maturity (day 3), all animals were transferred to new plates daily until the day of scoring. The day of scoring was selected to maximize detection of suppression or enhancement. Each experiment consists of at least three separate days of scoring that total 150 or more neurons when combined.

Overexpression and rescue lines. sbd-6::hda-1 (pHA442) was constructed by inserting hda-1 cDNA between the KpnI and SacI sites behind the sbd-6 promoter of plasmid pHA#355 (Baran et al., 1999). hda-1(e1795)/unc-76(e911);rtls18[Htn-Q150] animals were injected with 50 ng/μl sbd-6::hda-1 and 25 ng/μl myo-2::GFP (pPD48.33) (Thatcher et al., 1999) to rescue hda-1(e1795) polyglutamine toxicity enhancement. hda-1(e1795);Htn-Q150 homozygous animals carrying the extrosomal sbd-6::hda-1 transgene were selected and aged to 6 d before scoring for degeneration. As a control, hda-1(e1795)/unc-76(e911);rtls18[Htn-Q150] animals were injected with 50 ng/μl sbd-6::empty pHA#355 and 25 ng/μl myo-2::GFP (pPD48.33) and scored in parallel. rtls18[Htn-Q150] animals were injected with 50 ng/μl sbd-6::hda-1 and 25 ng/μl myo-2::GFP to create hda-1(0Ex);Htn-Q150 transgenic lines overexpressing hda-1. sbd-6::hda-3 (pHA401) rescue constructs were generated by insertion of hda-3 cDNA into pHA#355 plasmid between the KpnI and SacI sites and injected at 30 ng/μl sbd-6::hda-3 pHA401 with 120 ng/μl pha-1 pBX1#1 into hda-3::pha-1(0Ex);rtls11[Htn-Q150] animals. pha-1 rescue serves as a transformation marker. hda-3 overexpression lines were generated by injection of 100 ng/μl sbd-6::hda-3 and 120 ng/μl pha-1 pBX1#1 rescue construct in hda-3(+/+);pha-1[Htn-Q150] animals. At least three transgenic lines were scored and pooled for all genotypes.

Chemical inhibition of HDAC function. pha-1;rtls11 (Htn-Q150) L1 animals were raised in a humidified chamber at 25°C in S-basal medium with (2%) DMSO with or without 150 μg/ml trichostatin A (TSA) (Sigma, St. Louis, MO) for 8 d before scoring.

Quantitative PCR. Gravid adults were lysed to collect staged eggs. Embryos were hatched and synchronized to stage L1 larva by starvation overnight in M9 buffer (Johnson et al., 1984). Animals were plated on RNAi nematode growth media plates seeded with HT115 bacteria expressing double-stranded RNA of the desired target gene. Animals developed to young adults at 25°C. A subset of the adults was aged to confirm degeneration phenotypes. The remaining young adult animals were washed off plates with M9 buffer. After centrifugation, supernatant was replaced with 4× volume RNAzol (Iso-Tex Diagnostics, Friendswood, TX). Solution was vortexed and flash frozen in liquid nitrogen to thaw at 37°C, vortexed, and flash frozen again. This process was repeated twice before tubes were stored at −80°C. RNA was isolated using standard procedure by freeze/thaw in RNAzol and phenol/chloroform/isooamylophil-cel ect extraction. cDNA was generated using polya primers and Super II single-strand reverse transcription (RT)-PCR (Invitrogen, San Diego, CA). RNA was quantified using TaqMan quantitative PCR (PE Biosystems, Foster City, CA). Primers were designed to span sequence from two exons to prevent genomic DNA amplification. Htn-Q150 transcripts were amplified using primers: forward, CAAGGTTACAGCTCGACAGATC; reverse, CTCCTAAGGGTCTCCTTCGG. All primers anneal at 58 or 59°C. Transcript levels were detected using TaqMan Htn Probe: CCGGGAATTCG. Transcripts of two neurally expressed genes, osm-6 and cat-1, were measured as comparisons for loading controls (Perkins et al., 1986; Zhang et al., 2004). osm-6 primers are as follows: forward primer, GCCCGAAAATATAGGATACAAA, and reverse primer, CATATTCCTTCTGTCATACAAACCT, which anneals with a melting temperature of 59°C. osm-6 transcript levels were detected with TaqMan Probe: TCTATGTTCCCGAGATG. The reaction contained 5 μl template DNA and 50 μM primer DNA in 50 μl salt and 1 μl Mg2+ . A three-step PCR was performed for 35 cycles. Denaturation temperature was 94°C for 20 s. Annealing was performed at 55°C for 20 s. Extension was performed at 72°C for 30 s.

Statistical analysis. Error bars represent the SD for multiple determinations of each genotypic; each genotype was examined in independent experiments undertaken on more than 1 as indicated in the text. n ≥ 3 for each genotype. Pairwise analysis using the two-tailed t test or ANOVA were used as indicated to determine statistical significance. Microsoft Excel (Microsoft, Seattle, WA) was used for all statistical calculations.

Results

Htn-Q150 expression perturbations CBP-1/CRH-1 function

Previous studies have suggested that expanded polyglutamine tracts perturb CBP and/or CREB function, thereby contributing
to transcriptional dysregulation and eventual neuronal death. Based on these findings we hypothesized that decreasing CBP or CREB function in neurons expressing an expanded huntingtin protein should increase neurodegeneration. This hypothesis was tested in a C. elegans model of neuronal polyglutamine toxicity (Faber et al., 1999). The first exon of human huntingtin was expressed in a small subset of C. elegans neurons, including the two ASH sensory neurons. Each ASH neuron has a sensory process that directly contacts the environment allowing neurons to take up the lipophilic, fluorescent dye DiD. Perturbations in the sensory process prevent dye uptake.

Expression of expanded polyQ tracts leads to neuronal dysfunction, perturbations in ASH sensory processes, and, eventually, cell death. Therefore, dye filling is a rapid, sensitive assay that can detect either degeneration or neuronal death caused by polyQ toxicity; this study does not distinguish between them.

To test the hypothesis that transcriptional dysregulation contributes to polyglutamine toxicity, the requirement for cbp-1 function was examined. Each of the two deletion alleles of the C. elegans ortholog of CBP, cbp-1, were introduced to rtIs18[Htn-Q150] transgenic animals. cbp-1(bm1) generates a stable, truncated protein that lacks the conserved histone acetyltransferase (HAT) domain and bromodomain. The cbp-1(bm2) allele deletes the promoter and first four exons of cbp-1. cbp-1(bm2) is a complete loss of function deletion allele; no CBP-1 protein is detected at the promoter and first four exons of cbp-1 is a complete loss of function deletion allele; no CBP-1 protein is detected. Previous results suggesting that expanded polyglutamine tracts interfere with CBP-mediated transcription.

Chemical inhibition of class I and class II HDACs decreases degeneration

Pharmacological manipulation of HDACs alters polyQ toxicity. Sirtuins increase class III HDAC activity and suppress polyQ toxicity in many organisms. Broadly acting class I and class II chemical HDAC inhibitors decrease polyQ-mediated neurodegeneration in vertebrate and Drosophila neurons (McCampbell et al., 2001; Steffan et al., 2001; Hockly et al., 2003). We found that TSA, a chemical inhibitor of class I and class II HDACs, reduces degeneration in C. elegans neurons. pha-1(rtls11) [Htn-Q150] L1 animals were cultured for 8 d with or without TSA. Degeneration was reduced from 61 ± 9% in control animals to 26 ± 3% in animals incubated with 150 μg/ml TSA (n > 150 neurons; two trials) (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). TSA suppression was dose dependent; degeneration was moderately reduced by 100 μg/ml TSA (41 ± 6%; n = 100). These results confirm HDACs modulate polyQ toxicity in C. elegans, but broadly acting HDAC inhibitors cannot reveal the contributions of specific HDACs to polyQ toxicity.

Decreasing HDAC gene function has differing effects on Htn-Q150 toxicity

The activity of histone acetyltransferases, including CBP, is opposed by histone deacetylases. Each of the three HDAC classes is represented in C. elegans (supplemental Fig. 2 and supplemental Table 2, available at www.jneurosci.org as supplemental material). Class I HDACs include hda-1 (C53A5.3), hda-2 (C08B11.2), and hda-3 (R06C1.1) (Shi and Mello, 1998). Mammalian orthologs of C. elegans class I HDACs are ubiquitously expressed and predominantly localized to the nucleus. Previous analysis revealed a role for hda-1 in development. Homozygous hda-1(e1794) animals are defective in gonadogenesis and are, consequently, sterile (Dufourcq et al., 2002). C. elegans class II HDACs include hda-4 (C10E2.3), hda-5 (F43G6.11), hda-6 (F41H10.6), hda-10 (YS1H1A.5), and hda-11 (C35A5.9) (Dufourcq et al., 2002). C. elegans class III HDACs include sir-2.1 (R11A8.4), sir-2.2 (F46G10.7), and sir-2.3 (F46G10.3). Decreased function of sir-2.1 reduces lifespan, whereas overexpression of
HDACs, because mutant alleles were not available. Consistent degeneration (sir-2.1(OEx);Htn-Q150) expression in generation (79% sir-2.3). Guarente, 2001; Parker et al., 2005). Although complete loss of hda-4 reduces animals at day 8) (Fig. 2). In addition, when function of a previously described polyQ transgene, 47% of the ASH neurons degenerate by day 8. Degeneration is decreased to 20 ± 4% by increased expression of HDA-1 (50 ng/µl) in Htn-Q150 ASH neurons. Expression of HDA-1 does not cause detectable degeneration of ASH neurons that do not express Htn-Q150 (2 ± 2%). No significant change in neurodegeneration is detectable in animals that carry an empty sir-6 promoter and the transgenic marker myo-2::GFP (50 ± 10%). ***p < 0.0005 compared with corresponding Htn-Q150 animals in column 1; **p < 0.005 when comparing hdo-1;Htn-Q150; sir-6::hda-1 with hdo-1;Htn-Q150 in column 2 (to assess rescue in A). Empty promoter transgenes did not significantly change polyglutamine toxicity. At least three trials were conducted for each experiment to total n > 150. Error bars represent SD.

sir-2.1 increases lifespan (Tissenbaum and Guarente, 2001). The relative contribution of these HDACs to polyglutamine toxicity is unclear. Perturbation or modulation of individual HDAC genes could have different effects on polyglutamine toxicity. In C. elegans, gene function can be systematically reduced using either If alleles or RNAi. Only a few loss of function alleles exist for HDAC genes; RNAi knockdown was used to examine the other C. elegans HDAC genes. Although neurons are relatively insensitive to RNAi, we found that feeding animals bacteria expressing dsRNA reduced expression of genes in the ASH neurons. When animals were fed bacteria expressing GFP dsRNA, GFP expression was lost in 88% of the ASH neurons (n = 100), whereas all control animals expressed GFP in both ASH neurons. In addition, when function of a previously described polyQ enhancer-1 (pque-1) (Faber et al., 2002) was reduced by feeding pque-1 dsRNA, Htn-Q150-induced ASH neuron degeneration was increased from 26 ± 13% in control animals to 81 ± 9% (n = 100 for each). Thus, ASH neurons are susceptible to RNAi. Loss of function alleles and/or RNAi was used to examine the ability of each C. elegans HDAC gene to modulate polyglutamine toxicity.

Four HDAC loss of function alleles were tested for their ability to modify Htn-Q150 toxicity; three of the four enhanced toxicity. Heterozygous loss of hda-1 increased degeneration of ASH neurons in Htn-Q150 animals (86 ± 6 from 46 ± 4% in control animals at day 8) (Fig. 2A). Similarly, reducing function of a class II HDAC, hda-4, increased degeneration (65 ± 7%). Deletion alleles exist for two of the class III HDAC genes (Tissenbaum and Guarente, 2001; Parker et al., 2005). Although complete loss of sir-2.3 function had no effect on Htn-Q150-induced degeneration (48 ± 2%), complete loss of sir-2.1 function enhanced degeneration (79 ± 6%). Consistent with this result, sir-2.1 overexpression in sir-2.1(OEx);Htn-Q150 animals suppressed degeneration (23 ± 2%) (Fig. 2A).

RNAi was used to examine the contributions of remaining HDACs, because mutant alleles were not available. Consistent with preceding analysis, degeneration is increased to 66 ± 8% in hda-1(RNAi);Htn-Q150 animals (44 ± 7% in Htn-Q150 animals on control RNAi) (Fig. 2B). Similarly, RNAi of the class I HDAC, hda-2, and all class II HDACs increased degeneration (Fig. 2B) (quantified in supplemental Table 2, available at www.jneurosci.org as supplemental material). In contrast, hda-3(RNAi) reduced degeneration (20 ± 5%). RNAi of the class III HDAC, sir-2.2, increased degeneration to 81 ± 4%. Given the similarity between class I HDACs at the molecular level, the dramatically different effect of hda-3 knockdown is striking. To address the cellular specificity of class I HDACs on polyglutamine toxicity, additional analysis of hda-1 and hda-3 was undertaken.

HDA-1 activity protects Htn-Q150-expressing neurons hda-1(e1795) homozygous animals are sterile and die prematurely (Dufourcq et al., 2002). The overall poor health of homozygous hda-1(e1795) animals could contribute to the enhanced polyglutamine degeneration observed in hda-1(e1795); Htn-Q150 animals. To address this issue and to determine whether hda-1(IV) enhancement of polyglutamine toxicity was cell autonomous, an hda-1 cDNA was expressed in ASH neurons using the sir-6 promoter, which drives expression in a limited number of C. elegans neurons (Troemel et al., 1995). Degeneration in homozygous hda-1(e1795) animals was evaluated at day 6, because hda-1(e1795) homozygous animals rarely survive longer. In Htn-Q150 animals, 6 ± 2% of the ASH neurons degenerated by day 6 (Fig. 3A). Homozygous loss of hda-1 increases toxicity (36 ± 2%). Expression of the hda-1 CDNA in 6-d-old homozygous hda-1(e1795);Htn-Q150 animals restored neuronal survival to original levels (4 ± 3%) (Fig. 3A). Injection of equal concentrations of an empty sir-6 promoter plasmid, sir-6::empty, and the transgenic marker fail to rescue the homozygous hda-1(e1795) enhancement of polyglutamine toxicity (29 ± 2%). No ASH neurons degenerated in hda-1(e1795) homozygous animals lacking the Htn-Q150 transgene. We conclude that loss of hda-1 function is deleterious in neurons that express expanded polyglutamine proteins. Restoring hda-1 levels in a small subset of neurons, including the ASH neurons, is sufficient to ameliorate hda-1 loss of function enhancement of Htn-Q150 toxicity. This suggests that, normally, HDA-1 acts within the affected neuron and can protect against Htn-Q150 toxicity; reducing HDA-1 function sensitizes neurons to polyglutamine assault.
Decreasing hda-1 function could negatively affect neuronal survival by a nonspecific mechanism. However, if overexpression of hda-1 protects against Htn-Q150-induced neurodegeneration, then HDA-1 targets are likely directly involved in protecting polyglutamine-expressing neurons. To test this hypothesis, hda-1 was overexpressed in ASH neurons. Htn-Q150 animals were injected with srb-6: hda-1 to create hda-1 overexpression transgenic animals [hda-1(OEx); Htn-Q150]. Suppression is most readily detected by comparing degeneration in Htn-Q150 animals, Htn-Q150; hda-1(OEx) animals, and Htn-Q150; srb-6:empty transgenic control animals at day 8. In Htn-Q150 animals without the srb-6: hda-1 transgene, 47 ± 4% of the ASH neurons degenerate by day 8. Degeneration is decreased to 20 ± 4% by increased expression of HDA-1 in Htn-Q150 ASH neurons (Fig. 3B). Expression of HDA-1 does not cause significant degeneration in the absence of the Htn-Q150 transgene (1 ± 2%). Increased HDA-1 activity in the neurons protects them against Htn-Q150 toxicity, suggesting that HDA-1 targets may be critical factors in decreasing polyglutamine toxicity.

HDA-3 promotes degeneration in response to Htn-Q150 expression

The hda-3(RNAi) analysis suggested that reducing HDA-3 function could decrease Htn-Q150-induced degeneration. To confirm the role of hda-3 in Htn-Q150-induced neurodegeneration, a deletion allele, tm374, was obtained from the C. elegans Gene Knockout Center (Tokyo, Japan). The tm374 deletion begins in the second exon of hda-3, omits DNA sequence encoding the FPGTDGL residues that are critical for degacetyl activity (Finnin et al., 1999), and results in a frame shift that is likely to eliminate hda-3 function. The integrated rtls18[ Htn-Q150] array maps are very close to the hda-3 gene on chromosome I. Therefore, it was necessary to use a different Htn-Q150 transgene for hda-3(tm374) analysis. Degeneration was assessed in rtls11[ Htn-Q150] and rtls14[ Htn-Q150] animals on day 7 when either enhancement or suppression of Htn-Q150 toxicity could be detected. hda-3(tm374) recapitulated the suppression of Htn-Q150 toxicity observed in hda-3(RNAi) animals. Degeneration was reduced from 27 ± 2% in rtls11[ Htn-Q150] animals to 6 ± 5% in homozygous hda-3(tm374); rtls11[ Htn-Q150] animals (Fig. 4A).

To confirm that HDA-3 acts in the polyglutamine expressing neurons to modulate toxicity, we examined the HDA-3 cellular expression pattern. Immunohistochemistry revealed that HDA-3 is ubiquitously expressed. Coating with an OSM-10 antiserum that recognizes an endogenous protein expressed in the ASH (Hart et al., 1999) confirmed that HDA-3 protein is found in the ASH neurons. Endogenous HDA-3 predominantly localizes to the nucleus in C. elegans, where it is likely to function in transcriptional regulation (supplemental Fig. 3, available at www.jneurosci.org as supplemental material) (Whetstine et al., 2005).

To determine whether hda-3 modulation of Htn-Q150 toxicity occurs in the neurons and to confirm that reduced hda-3 function is responsible for suppression, an srb-6:: hda-3 transgene was introduced into hda-3(rtls11)[ Htn-Q150] animals. Polyglutamine-induced degeneration of ASH neurons was restored when hda-3 was expressed in a small subset of neurons, including ASH. Degeneration averaged 31 ± 8% in hda-3(tm374); rtls11[ Htn-Q150]; srb-6:: hda-3(+) transgenic animals compared with 27 ± 2% in rtls11[ Htn-Q150] control animals and 10 ± 3% in hda-3(rtls11)[ Htn-Q150] animals (Fig. 4A). Thus, decreased HDA-3 activity decreases the toxicity of Htn-Q150 specifically in neurons in which polyglutamine is expressed.

To determine whether HDA-3 contributes directly to Htn-Q150-induced degeneration in ASH neurons, srb-6::hda-3 was introduced into Htn-Q150 animals that retain normal hda-3 activity. Overexpression of hda-3 in ASH using the srb-6 promoter increased degeneration from 24 ± 11 to 45 ± 13% (Fig. 4B). We concluded that suppression of polyglutamine toxicity in hda-3(tm374); rtls11[ Htn-Q150] animals is attributable to loss of hda-3 function and that hda-3 acts cell autonomously to modulate polyglutamine toxicity in these neurons.

Loss of hda-3 or hda-1 specifically modulates polyglutamine-induced neurotoxicity

To determine whether hda-1 or hda-3 modulation of neurotoxicity is specific to polyglutamine-induced degeneration, we examined possible roles of hda-1 and hda-3 in necrotic neurodegeneration independent of polyglutamine toxicity. Hyperactive ion channels disrupt cellular osmotic balance and cause necrotic neuronal degeneration. A recessive, cold-sensitive mutation in a sodium channel, deg-1(rt70), causes temperature-dependent ASH degeneration (Faber et al., 2002). hda-1 and hda-3 functions were individually reduced by RNAi in deg-1(rt70) mutant animals. deg-1(rt70) animals were aged to 4 d at 20°C when 9 ± 4% of the ASH neurons degenerate or were aged to 7 d at 15°C when 78 ± 6% of the ASH neurons degenerate to assess enhancement or suppression, respectively. Neither hda-1(RNAi) nor hda-3(RNAi) altered necrotic degeneration (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). These data suggest that transcriptional dysregulation selectively contributes to polyglutamine-induced degeneration.

Reducing function of hda-3 or hda-1 does not significantly alter Htn-Q150 expression

Reducing function of specific proteins found in HDAC complexes can alter transgene expression levels in many C. elegans tissues, but neurons are generally protected from these alterations (Kim et al., 2005). To address the possibility that Htn-Q150 transgene levels were changed by loss of HDAC function in the ASH neurons, we took three experimental approaches. First, to demonstrate that osm-10:: Htn-Q150 transgene levels are not altered by hda-3 loss of function, we quantified expression of osm-10::GFP in homozygous hda-3(tm374); osm-10::GFP animals. GFP expression in ASH neurons was indistinguishable.
from control osm-10::GFP animals (n = 380). To directly determine whether genes implicated in transgene silencing decrease osm-10 promoter activity, we examined GFP expression in 3-d-old rts11[osm-10::GFP, osm-10::Htn-Q150] animals lacking an RNA-directed RNA polymerase, rrf-3, or lacking a ribosomal RNA repressor, ncl-1 (Frank and Roth, 1998; Simmer et al., 2002). Both of these genes are expressed in ASH neurons and have been implicated previously in transgene silencing in C. elegans (Simmer et al., 2002; Kim et al., 2005). Neither rrf-3(pk1426) nor ncl-1(e1865) silence expression of GFP in ASH neurons (n = 200 and n = 150, respectively). Additionally, to determine specifically whether rrf-3(pk1426) or ncl-1(e1865) could suppress or enhance polyglutamine-induced degeneration, ASH neuron degeneration was assessed in corresponding animals. Degeneration was not altered in rrf-3 or ncl-1 homozygous mutant backgrounds (supplemental Fig. 5A, available at www.jneurosci.org as supplemental material). Therefore, we consider it unlikely that transgene silencing contributes to decreased neurodegeneration in hda-3 mutant animals.

To directly address the possibility that reducing HDAC function alters Htn-Q150 expression levels, consequently altering polyglutamine toxicity, Htn-Q150 mRNA levels were measured using quantitative RT-PCR. Transcript levels were measured in hda-3(RNAi), hda-1(RNAi), and control RNAi rts18[Htn-Q150] animals. Levels were assessed in young adult animals before the onset of neuronal degeneration. There was no significant difference in Htn-Q150 mRNA levels (supplemental Fig. 5B, available at www.jneurosci.org as supplemental material). We conclude that changes in Htn-Q150 toxicity are not attributable to changes in Htn-Q150 transcript levels.

HDA-3 loss suppresses CREB modulation

Htn-Q150-induced degeneration

Histone deacetylases are critical for transcriptional regulation, but HDACs are not limited to histones as targets (Hubbert et al., 2002). C. elegans HDACs could modulate polyglutamine toxicity via transcriptional regulation or by deacetylating proteins that are directly involved with polyglutamine toxicity. Genetic epistasis analysis was undertaken to determine whether hda-3(sc1374) suppression of Htn-Q150 toxicity is via regulation of CREB homolog (C. elegans CREB homolog) act in the same pathway, either the phenotype of hda-3 (suppression) or the phenotype of crh-1 (enhancement) should predominate. 28 ± 13% of the ASH neurons degenerate in control Htn-Q150 animals. As expected, degeneration increased to 59 ± 7% in crh-1/Htn-Q150 animals and decreased by loss of hda-3 to 12 ± 7% in Htn-Q150 animals (Fig. 5). Interestingly, hda-3 loss of function suppressed the crh-1 loss of function phenotype in hda-3(sc1374);crh-1/Htn-Q150 animals to 17 ± 8%. Thus, hda-3 is epistatic to crh-1, suggesting that HDA-3, directly or indirectly, opposes crh-1 activity in regulating transcription of genes that protect against Htn-Q150-induced toxicity.

Suppression of Htn-Q150 toxicity in hda-3 mutant animals requires HDA-1 function

Reducing hda-1 function enhances Htn-Q150 toxicity. In contrast, reducing hda-3 function suppresses Htn-Q150 toxicity. To determine whether hda-3 loss of function could suppress hda-1 enhanced polyglutamine toxicity, ASH neuron degeneration was assessed in hda-3/hda-1/++;Htn-Q150 animals. As expected, degeneration was reduced from 45 ± 4% in control rts14[Htn-Q150] animals to 29 ± 2% in hda-3/rrts14[Htn-Q150] animals at day 7. Consistent with results described above, degeneration was increased in hda-1/++;rrts14[Htn-Q150] animals to 76 ± 6% (Fig. 6). Interestingly, degeneration was also increased in hda-3(sc1374);hda-1(e1795)/++;rrts14[Htn-Q150] animals to 74 ± 7% versus controls. hda-3 loss of function can suppress degeneration when hda-1 function is intact but cannot suppress Htn-Q150-induced degeneration when hda-1 levels are compromised. One possible interpretation of these results is that HDA-1 activity is required, directly or indirectly, for suppression of Htn-Q150 toxicity by hda-3 loss of function.

Discussion

This analysis suggests that expression of expanded Htn fragments interferes with CBP and/or CREB function, thereby contributing to neuronal degeneration. Reducing function of most C. elegans histone deacetylases enhances Htn-Q150-induced degeneration. Additional analysis confirms that HDA-1 protects Htn-Q150 expressing neurons from degeneration. HDA-3 is unique; loss of HDA-3 suppresses polyglutamine toxicity.
Reducing cebp-1 and crh-1 activity enhances Htn-Q150-induced degeneration. This suggests that expanded polyglutamine perturbs transcription leading to neuronal degeneration. Histone acetyltransferase and Q-rich domains of human CBP interact with expanded Htn (Steffan et al., 2001). *C. elegans* cebp-1(bm1), the allele that deletes the histone acetyltransferase and bromodomains, significantly enhances polyglutaminicity. cebp-1(bm1) encodes a stable, truncated CBP-1 protein that lacks acetyltransferase activity (Víctor et al., 2002). This suggests that acetyltransferase activity is important for CBP-1 protection against Htn-Q150 toxicity.

Reducing crh-1 (CREB) function is sufficient to enhance polyglutaminicity, implicating crh-1 regulated genes in neuronal survival. Deletion of CREB combined with deletion of its cofactor, CREM (cAMP-responsive element modulator), causes a phenotype similar to Huntington’s disease in mice (Mantamadotis et al., 2002). However, reducing *C. elegans* cebp-1 or eliminating crh-1 function does not cause degeneration independent of the Htn-Q150 transgene. We conclude that Htn-Q150 perturbs additional transcriptional pathways and/or cellular processes that contribute to degeneration. In vertebrates, TATA binding protein, specificity protein 1, and p53 have all been found in polyglutamine aggregates (Cha, 2000; Sugars and Rubinsztein, 2003). In addition, proteasomes, ubiquitin, and chaperones have been found in aggregates, suggesting disruption of the protein folding and degradation pathways (Sakahira et al., 2002; Ciechanover and Brundin, 2003). The conclusion that multiple pathways contribute to polyglutaminicity is strengthened by the recent finding that protective effects of increased CREB and heat-shock protein 70 are additive in *Drosophila* (Iijima-Ando et al., 2005). We conclude that histone acetylation activity of CBP-1 and transcriptional activation by CRH-1 are likely perturbed by Htn-Q150 expression contributing to the process of neurodegeneration.

Chemical and genetic manipulation of HDAC function yield distinctly different results for polyQ toxicity. This likely reflects their different specificity and efficacy. polyQ toxicity is reduced by broadly acting chemical inhibitors of class I and class II HDACs. In contrast, eliminating specific HDACs can increase or reduce degeneration depending on the gene targeted. At first glance, this discrepancy may be puzzling. However, at the concentrations tested, broadly acting chemical inhibitors like TSA likely cause a partial decrement in all class I and class II HDACs. These HDACs likely regulate many genes with various and divergent impacts on polyQ toxicity. It is reasonable to assume that eliminating specific HDACs using genetic techniques might then yield dramatically different results; loss of a specific HDAC likely yields specific and unique changes in gene expression (Dangond and Gullans, 1998). Additionally, chemical or genetic manipulation may differ in their impact on the dispensatory mechanisms that operate when HDAC function is decreased (Ajamian et al., 2004). And, chemical inhibitors may have off-target effects, perturbing proteins other than HDACs. Previous analyses using broadly acting chemical inhibitors may have obscured the relative importance of specific HDACs in polyQ toxicity.

Decreasing the function of most class I and class II HDACs increased polyglutaminicity in *C. elegans*. Mammalian HDAC1 and HDAC2 are found together in numerous complexes [Sin3, v粗ose remodeling and deacetylation, retinoblastoma, and corepressor to RE1 silencing transcription factor/neural restrictive silencing factor corepressor complexes (Knoopfer and Eisenman, 1999; Humphrey et al., 2001)]. In mammals, Sin3 is important for the regulation of fatty acid synthesis, which is critical for mitochondrial function and vesicle formation, both of which have been implicated in polyglutamine expansion disease (Schweizer and Hofmann, 2004). Sin3 and nuclear repressor protein (NCoR) interact with Htn when the polyglutamine tract is expanded and have been found in polyglutamine protein aggregates (Bourett et al., 1999). Although mammalian HDAC3 is found in complexes with NCoR and SMRT (silencing mediator of retinoic acid and thyroid hormone receptor), it is not yet clear in which complexes *C. elegans* hda-3 takes part (Guenther et al., 2000, 2001; Li et al., 2000; Wen et al., 2000). If ectopic polyglutamine interactions with Sin3 and NCoR titrate away functions of these repressor complexes, then reducing the function of HDACs in the Sin3 and NCoR complexes could further decrease repressor complex function and thereby impinge on neuronal survival. In this study, reducing function of most class II HDACs increases polyglutaminicity. Many class II HDACs have myocyte enhancer factor-2 (MEF-2) interaction domains (Choi et al., 2002). Mammalian MEF-2 proteins play important roles in neuronal survival (Mao and Wiedmann, 1999; Yoon et al., 1999). Thus, decreasing function of class II HDACs could compromise survival of neurons. HDAC4 and HDAC5 repression of MEF-2 is dependent on calcium, suggesting a possible link between calcium signaling and polyglutaminicity (Yoon et al., 2000). Thus, *C. elegans* HDA-1, HDA-2, and class II HDACs could be important for maintaining survival of neurons under polyglutamine stress.

In contrast, loss of *Ce-hda-3* suppresses polyglutaminicity. Mammalian HDAC3 is found in different complexes from HDAC1 and HDAC2 (Guenther et al., 2000, 2001; Wen et al., 2000). Thus, specific HDAC complexes could regulate genes critical for protection against polyglutaminicity or neuronal survival, whereas other complexes regulate genes that contribute to polyglutaminicity.

Class III HDACs have been implicated previously in polyglutaminicity and aging. In *C. elegans* and *Drosophila*, increased Sir2 function extends the lifespan (Tissenbaum and Guarente, 2001; Wood et al., 2004). In this study, reducing function of two of the three *C. elegans* class III HDACs enhanced polyglutaminicity. Interestingly, *Ce-sir-2.1* deletion increases Htn-Q150-induced degeneration, whereas overexpression suppresses degeneration. Our results are consistent with recent findings that increasing activity of *Ce-sir-2.1* protects against polyglutamine-induced neuronal dysfunction (Parker et al., 2005). Based on the results presented herein, *Ce-sir-2.1* is not unique. Decreasing function of most *Ce*-HDACs increases polyglutaminicity, and increasing both *Ce-hda-1* and *Ce-sir-2.1* suppresses toxicity. The results presented here suggest that SIR-2.1 HDAC activity is critical for polyglutaminicity but not necessarily increased longevity or aging associated with activity of *sir-2.1*.

Is the activity of *Ce-hda-3* as a transcriptional regulator critical for its role in polyglutaminicity? Some HDACs target proteins that are not involved in transcription. For instance, mammalian HDAC6 and Sir2 deacetylase tubulin (Hubbert et al., 2002; Matsuyama et al., 2002; North et al., 2003; Zhang et al., 2003). Therefore, at least two functional targets exist for HDACs in polyglutaminicity. First, specific HDACs could regulate the transcription of genes that encode protective proteins or proteins that assist in degeneration. Second, specific HDACs could deacetylate proteins that play nontranscriptional roles underlying polyglutaminicity. Based on the ability of *Ce-hda-3* deletion to suppress increased polyglutaminicity of crh-1 mutants, genetic epistasis suggests that HDA-3 modulates polyglutaminicity through transcriptional regulation. *crh-1*
loss of function can no longer enhance polyglutamine toxicity in hda-3 mutant animals, suggesting that other transcriptional activators are able to compensate for 

chrl-1 function of hda-3 if HDA-3 might normally repress transcription of C. elegans genes that protect against expanded polyglutamine or promote neuronal survival. Therefore, release from negative regulation via hda-3 loss of function protects against polyglutamine toxicity.

Here, we demonstrate that normal Ce-hda-1 function is required for Ce-hda-3(tm1374) suppression of Htn-Q150-induced neurodegeneration. There are at least two molecular mechanisms that could underlie alterations in polyglutamine toxicity. Ce-hda-1 protein could negatively regulate cell death genes, and/or HDA-3 could repress transcription of protective genes. One possible interpretation of the epistasis analysis is that Ce-hda-1 target genes are downstream of Ce-HDA-3 targets. A second possible explanation is that Ce-hda-1 transcription may normally be directly repressed by Ce-HDA-3 protein in ASH neurons. Therefore, when Ce-hda-3 function is reduced, Ce-hda-1 transcription is increased. Our results demonstrate that increased Ce-hda-1 dosage suppresses Htn-Q150-induced neurodegeneration. Therefore, Ce-hda-3 loss of function could suppress polyglutamine toxicity by increasing HDA-1 activity. Thus, in Ce-hda-3(tm1374);Ce-hda-1(e1795);rts1[14][Htn-Q150] animals, neurodegeneration may not be suppressed, because Ce-hda-1 activity cannot be sufficiently increased. However, we cannot rule out the possibility that hda-3(lf) cannot compensate for the severity of hda-1(lf) degeneration.

In C. elegans, genes positively regulated by CREB/CBP and negatively regulated by hda-3 likely play important roles protecting against expanded polyglutamine. We speculate that in mammals, specific HDACs could negatively regulate transcription of bcl-2, BDNF, chaperones, or other proteins necessary for neuronal survival, whereas other HDACs regulate transcription of degeneration promoting genes such as caspases.

In summary, using a C. elegans neuronal model of polyglutamine disease, we found that CBP and CREB functions are critical for protecting against polyglutamine-induced neurodegeneration. Results presented here suggest that HDACs play opposing roles in polyglutamine toxicity in C. elegans neurons. Therefore, it is likely that in mammals, specific HDACs will also have opposing effects on polyglutamine-induced degeneration. These C. elegans results suggest that finding downstream target genes of specific HDAC complexes will help to determine the mechanism of polyglutamine-induced degeneration. The development of inhibitors targeted to specific HDACs or HDAC complexes could be both more effective and less toxic therapeutic agents in polyglutamine expansion diseases.

References
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